Alternate primers for whole-genome SARS-CoV-2 sequencing

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Abstract

As the world is struggling to control the novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), there is an urgency to develop effective control measures. Essential information is encoded in the virus genome sequence with accurate and complete SARS-CoV-2 sequences essential for tracking the movement and evolution of the virus and for guiding efforts to develop vaccines and antiviral drugs. While there is unprecedented SARS-CoV-2 sequencing efforts globally, approximately 19 to 43% of the genomes generated monthly are gapped, reducing their information content. The current study documents the genome gap frequencies and their positions in the currently available data and provides an alternative primer set and a sequencing scheme to help improve the quality and coverage of the genomes.

Keywords: SARS-CoV-2; COVID-19, primers; next generation sequencing;

Introduction

Since the first report on 30th December 2019 in Wuhan China and the WHO declaration of the pandemic on 12th March 2020, the novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) (1) and the associated disease Coronavirus Disease 2019 (COVID-19) (2)(3) have continued to spread throughout the world, causing >46 million infections and >1,200,000 death globally (4). The virus genome sequences carry important information, which can be used to interpret the virus transmission, evolution patterns and origin tracing. Furthermore, accurate and complete genomic sequences are essential for monitoring diagnostics and developing novel therapeutics and vaccines. We have seen an unprecedented amount of virus sequencing with over 130,000 complete or nearly complete genome sequences of SARS-CoV-2 now available in the GISAID database by the end of September 2020 (5). Most of the sequences have been generated by next generation sequencing using targeted amplicon methods. A scan through SARS-CoV-2 genomes from GISAID with the filter "complete genome" revealed a high frequency of gaps occurring across the genome, influencing the overall genomics quality and interpretation. Here we describe an alternate primer scheme for whole-genome sequencing to improve the genome sequence quality and coverage.

Documenting the problem

We retrieved genomes deposited to GISAID in September 2020 (9 months into the pandemic), using the “complete genome” filter and sorting the genomes by sequencing platforms information included in the metadata. Figure 1 illustrated the positions across the 30kb genome of every stretch of 200 Ns (N200; nearly the size of an amplicon) in the first genomes deposited in September 2020 using the Illumina platform (Panel A; N = 3000) versus MinION platform (Panel B; N = 3000). Histograms of gap frequencies across the genomes are shown for each platform. The gaps are not randomly distributed but occur with higher frequency in a subset of positions across the genome. Although genomes generated by the two platforms (Illumina and MinION) show similar problem regions (nt 8000-11000, and nt

19,000-24,000 relative to the reference genome NC_045512), the patterns are not completely identical. Given the use of several primer amplification schemes, we suspect the gaps in coverage may be due to unexpected primer interactions, complicated sequence regions (odd composition or secondary structure), issues with primer trimming during quality control of read data, or some combinations of these factors.

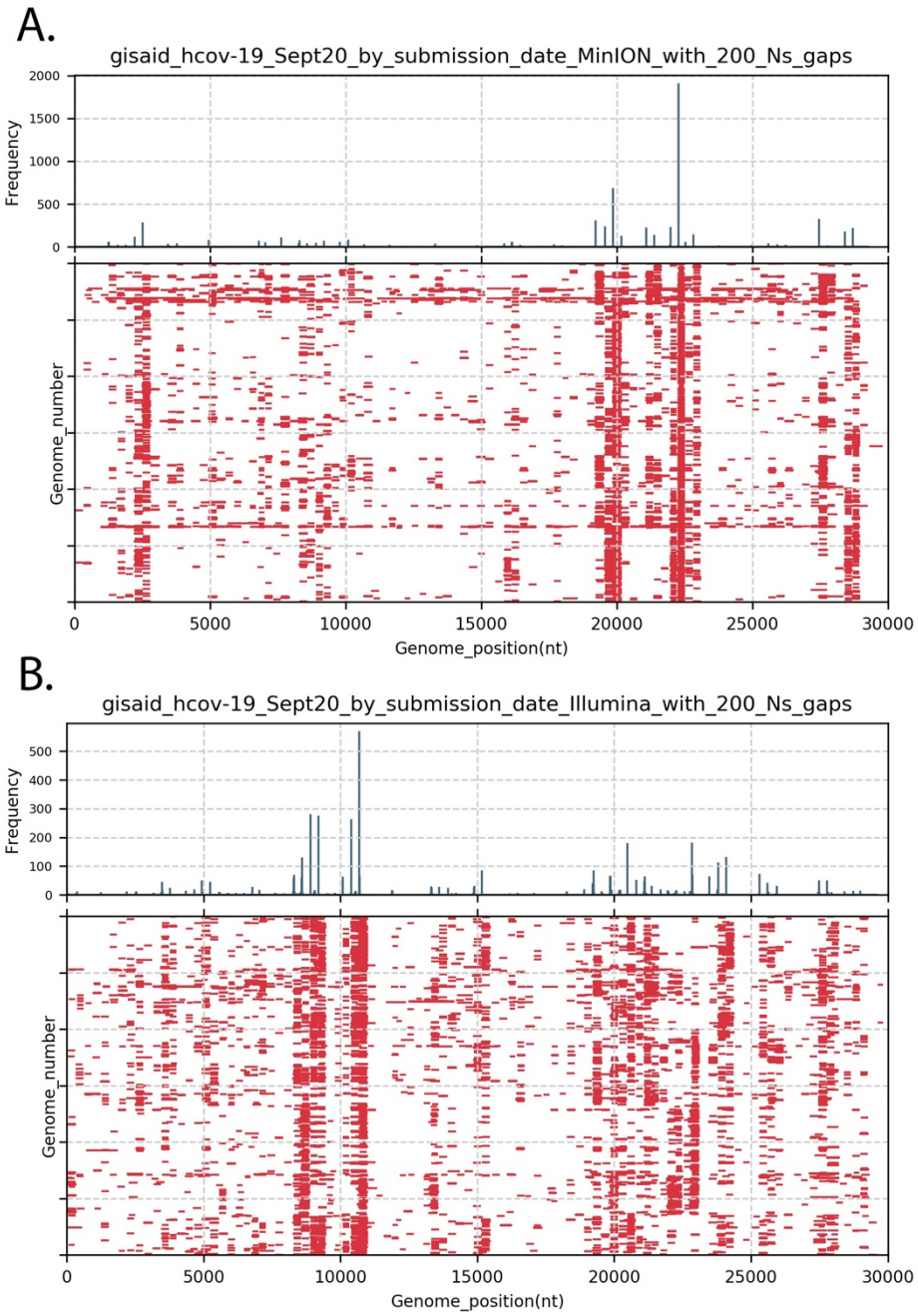

Figure 1. (updated graphics) **Positions of 200nt gaps across SARS-CoV-2 genomes listed as complete in GISAID**. Genomes deposited in September 2020 (n= 38,228) were retrieved from GISAID, sorted by sequencing platform (MinION versus Illumina) and genomes with at least 1 instance of 200N were collected. Panel A present gaps in the first 3000 MinION-generated genome sequences deposited that contained at least on 200N motif. Gaps >=200nt in each genome are indicated with red bars. The upper panel histogram shows the frequency (in 30 nt bins) of gaps >=200nt motifs by start position on genomes. Panel B is the same analysis of the first 3000 Illumina-generated genome sequences in September 2020 that contained at least one 200N motif.

The phenomenon is unlikely to be due to an isolated set of genomes as we observed similar N200 frequencies in genomes submitted from each month of the pandemic (Table 1), suggesting that gaps in coverage is a more general phenomenon. Of note, genomes generated using Ion Torrent show much lower levels of N200 (Table 1). The very low frequency of large gaps in the Ion Torrent data may be due to the use of a dedicated alternative primer set (6). There have been discussions and reports on the SARS-CoV-2 genome changes due to sequencing errors as well as long gaps in the genomes due to missing amplicons from the amplicon-approach sequencing (7) (8). Updates of the ARTIC primers have been presented in late March 2020 to address these issues (9) (10). Additional reports of longer amplicon methods have been published (11) (12) (14) including methods to use a subset of ARTIC primers to generate longer amplicons(13).

However, the percentage of reported complete genomes in GISAID with 1 or more N200s continues, with 10,611 (28%) of the 38,228 genomes deposited in September 2020 having 1 or more N200 gaps (Table 1), indicating the challenges remain largely unsolved.

Table 1. Frequency of SARS-CoV-2 genomes with 1 or more 200 nt gaps (N200) by month and by sequencing platform.

Deposition period	Complete genomes	Genomes with 1 or more N200 [2]	%. with 1 or more N200 [3]	Illumina total [4]	Illumina % with N200 [5]	MinION total [6]	MinION % with N200 [7]	Ion Torrent total [8]	Ion Torrent % with N200 [9]	Method unclear [10]	Method unclear % with N200 [11]
1Jan20-31Jan20	54	1	2	9	0	2	0	0	0	43	2
1Feb20-29Feb20	126	2	2	44	0	9	0	5	0	65	4
1Mar20-31Mar20	2872	559	19	1518	16	548	32	35	6	771	18
1Apr20-30Apr20	12411	3745	30	4970	38	1286	27	264	0	5424	26
1May20-31May20	19787	8606	43	8634	52	2634	30	529	0	7990	42
1Jun20-30Jun20	21665	8723	40	7043	36	3844	35	629	2	10149	47
1Jul20-31Jul20	17986	4834	27	4965	23	1585	33	471	2	10965	29
1Aug20-31Aug20	17276	4005	23	11074	22	2270	26	486	0	3446	28
1Sep20-30Sep20	38227	10611	28	22740	23	7973	44	580	1	6934	28

1. Number of genomes with the annotation "complete" retrieved from GISAID (https://www.gisaid.org/).

2. Genomes were sorted by the presence or absence of the sequence N200.

3. ((The number of genomes with at least 1 N200)/total number of genomes)*100.

4. Number of genomes in GISAID for this period generated using any of the Illumina methods as noted in the GISAID "Sequencing technology metadata" .

5. ((The number of Illumina genomes with at least 1 N200)/total number of genomes)*100.

6.,7. Number of genomes in GISAID for this period generated using any of the MinION methods as noted in the GISAID "Sequencing technology metadata" and their percentage.

8.,9. Number of genomes in GISAID for this period generated using any of the Ion Torrent methods as noted in the GISAID "Sequencing technology metadata" and their percentage.

10.,11. Number of genomes in GISAID for this period generated using unclear methods as noted in the GISAID "Sequencing technology metadata" and their percentage.

Detailed analysis of gaps.

A more focused analysis of the frequent gaps is provided in Figure 2. The gap pattern between nt 19,000 and 24,000 (relative to the reference genome NC_045512) is shown for both MinION and Illumina sequences (first 3000 genomes of each deposited in September 2020 with at least 1 200N motif). For reference the positions of the ARTIC primers (v.1) in the region are indicated (middle panel). A histogram of gap start positions (top panel) and the individual genome gaps (bottom panel) are also shown.

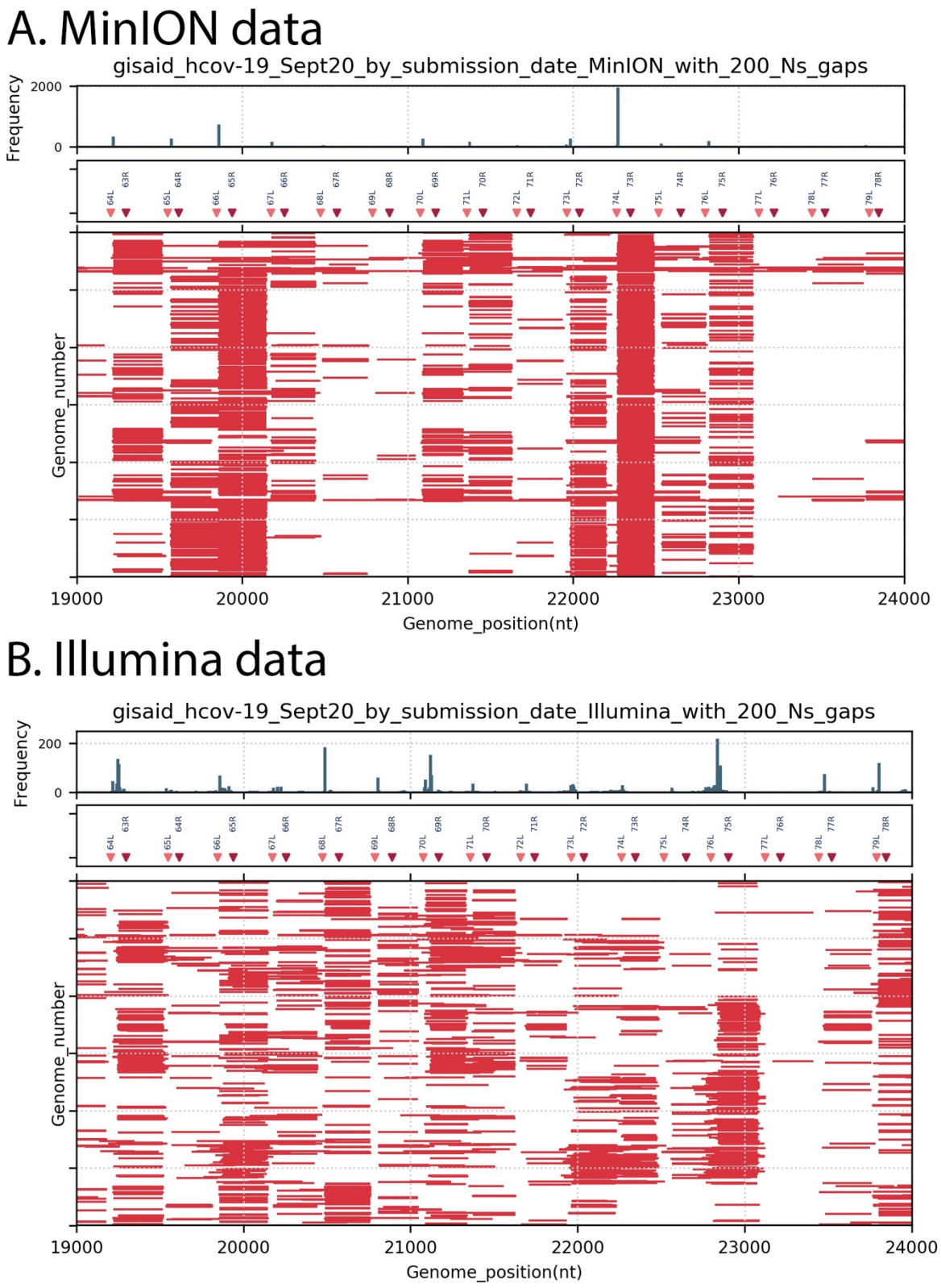

Figure 2. Positions of 200nt gaps across SARS-CoV-2 genomes stratified by MinION or Illumina, in region nt 19000 to 24000. Genomes deposited in September 2020 as “complete" were retrieved from GISAID, sorted by sequencing platform and by the presence of at least

one N200 motif. For clarity only the first 3000 genomes in each set were plotted. Similar to Figure 1, gaps >=200nt in each genome are indicated with red bars. The upper panel histogram shows the frequency (in 30 nt bins) of gaps >=200nt motifs by start position on genome, the middle panel plots the positions of ARTIC v.1 primers in the region(pink = forward "left" primers, red= reverse "right" primers) . Panel A: MinION-derived genome sequences, Panel B: Illumina-derived genome sequences.

The peaks of gap start positions frequently lie between forward primerL from Amplicon n and reverse primerR from Amplicon n-1, for both MinION and Illumina data. Because of overlapping amplicons commonly used, if a single amplicon is missing from the sequencing library (amplicon 74 for example), the resulting gap in coverage would not be the complete amplicon 74 but would span from the 3' end of the adjacent amplicon 73 (after primer and quality trimming) to the 5' end of adjacent amplicon 75 (after primer and quality trimming). The calculated gaps generated by such amplicon loss have a median length of 270.5 nt, which is close to the the observed median gap length in the MinION data (258 nt) or Illumina data (262 nt) from September 2020. This arrangement is outlined in the Supplementary Material Figure 1, Panel A.

Alternate primers as a potential solution to avoid gapped genomes.

We explored an alternate set of amplification primers (termed the **Entebbe primers**) designed using methods we had previously used for MERS-CoV (15), Norovirus (16), RSV (17) and Yellow Fever virus (18). Important for the design were the amplicon size and the primer placement. For their implementation, the use of primers for the reverse transcription step, and the multiplexing of the amplicons in two staggered sets was important for the PCR. Our experience had suggested an optimum amplicon size of around 1500 bp. The larger amplicons reduced the total primers content of the reactions but still allowed high reverse transcription efficiency (which, in our hands, declined beyond 1500 nt). Here we describe

primers designed for whole-genome sequencing of SARS-CoV-2, as well as sharing the detailed laboratory methods that we used for reverse transcription, PCR amplification and MinION library preparation to successfully sequence the SARS-CoV-2 genome.

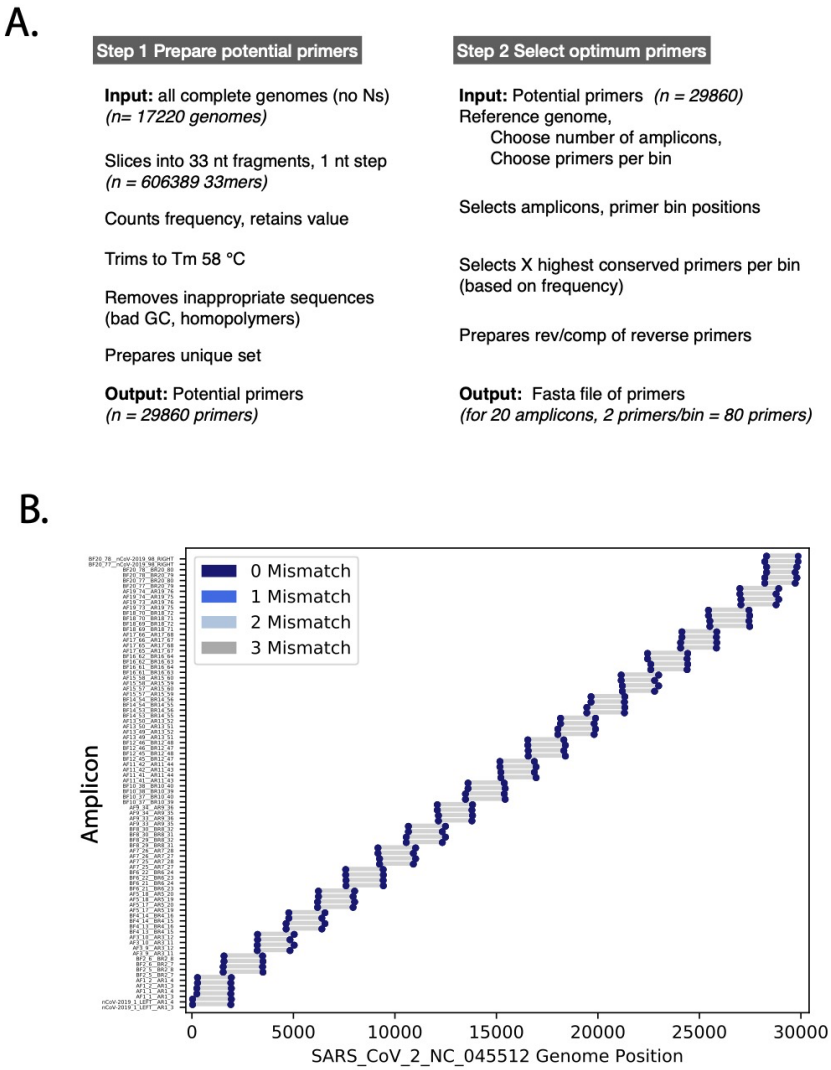

Figure 3. Primer design and amplicon layout. Panel A. The two main steps involved in primers generation and selection are shown. Panel B. The layout of the 20 amplicons across the SARS-CoV-2 genome is shown in lower panel. The blue markers indicate target positions in the SARS-CoV-2 genome (NC_045512 used here), the grey bars indicate the resulting amplicon.

Briefly, the primer design (Figure 3A) started with the set of complete SARS-CoV-2 genome sequences available in the GISAID database on 22 June 2020 (N = 21,687). Spaces and disruptive characters were removed from the sequence IDs and the sequences were further screened to remove genomes containing gaps of 6Ns or more, resulting in 17,220 clean genome sequences. Next, all sequences were sliced into 33nt strings (33mers), with a 1nt step and 606,389 unique 33mers were generated. The frequency of each 33mer was counted to identify highly conserved 33mers. This counting method avoids the multiple sequence alignment step commonly used in primer design and becomes prohibitive with large and or diverse genome sets. This alignment-free approach allowed us to use all suitable genome sequences of interest rather than a set that could be conveniently aligned. Finally, primer-like 33mers sequences were generated by trimming the sequences to a calculated desired melting temperature and removing any primers greater than 26nt.

In the second step we defined forward and reverse primer target regions (bins) for the amplicons. For SARS-CoV-2, we selected 20 amplicons with an overlap of 300nt, regularly spaced across the SARS-CoV-2 genome sequence (Figure 3B). We then selected the top conserved primer sequences (the highest frequency primers) mapping in the 5' or 3' 185nt of each amplicon. For security, the two highest frequency primers per bin were selected for the SARS-CoV-2 sequence, this provided some insurance against primer failure either due to target evolution or unexpected secondary structure. The binning and primer target locations for the final set of primers are shown in Figure 2 and the final calculated amplicon lengths were 1495-2093nt.

The reverse transcription, PCR amplification and library protocols were modified to accommodate the new primers. Important changes to note are the following. Reverse transcription was performed using the reverse primers and reverse transcription at 42°C. The PCR cycling conditions (using Phusion enzyme) were adjusted for the new T_ms and an increased elongation time required for the longer PCR products. Finally, the library purification

steps were adjusted to recover longer PCR and library products. A detailed step-by-step protocol is provided in the supplementary material.

Testing the performance of primers to sequence SARS-CoV-2 using MinION

We tested the Entebbe primers performance for sequencing SARS-CoV-2 from nucleic acid extracted from positive samples. The amplicon sizes and genome coverage are summarised in Figure 4. In particular, panel A and B (Figure 4) illustrate the amplicon products after the reverse transcription and PCR amplification with the expected sizes of 1400-2000 bp. These amplicons were then pooled and used for library preparation using the MinION sequencing kits SQK-LSK109. Final libraries were quantified and sequenced using a MinION Flow Cell (R9.4.1). The resulting read data, after quality and primer and adapter trimming, were then mapped to the SARS-CoV-2 Wuhan1 reference genome NC_045512 (Figure 4, panels C and D) to document sequence coverage across the genome. The twenty individual amplicons are detected in the coverage pattern with small peaks appearing where amplicons overlap. The coverage is consistent across the genome with no missing amplicons and the data were readily assembled into good coverage full genomes. Initial experiments showed that amplicons 2 (spanning nt 2,400) and 16 (spanning nt 23,500) had reduced yields (Figure 4, panel C). The primer mixes were subsequently adjusted to increase concentrations of amplicon 1 and 16 primers for reverse transcription and PCR (see detailed protocol in Supplementary materials), this improved yields relative to the other amplicons (Figure 4 panel D).

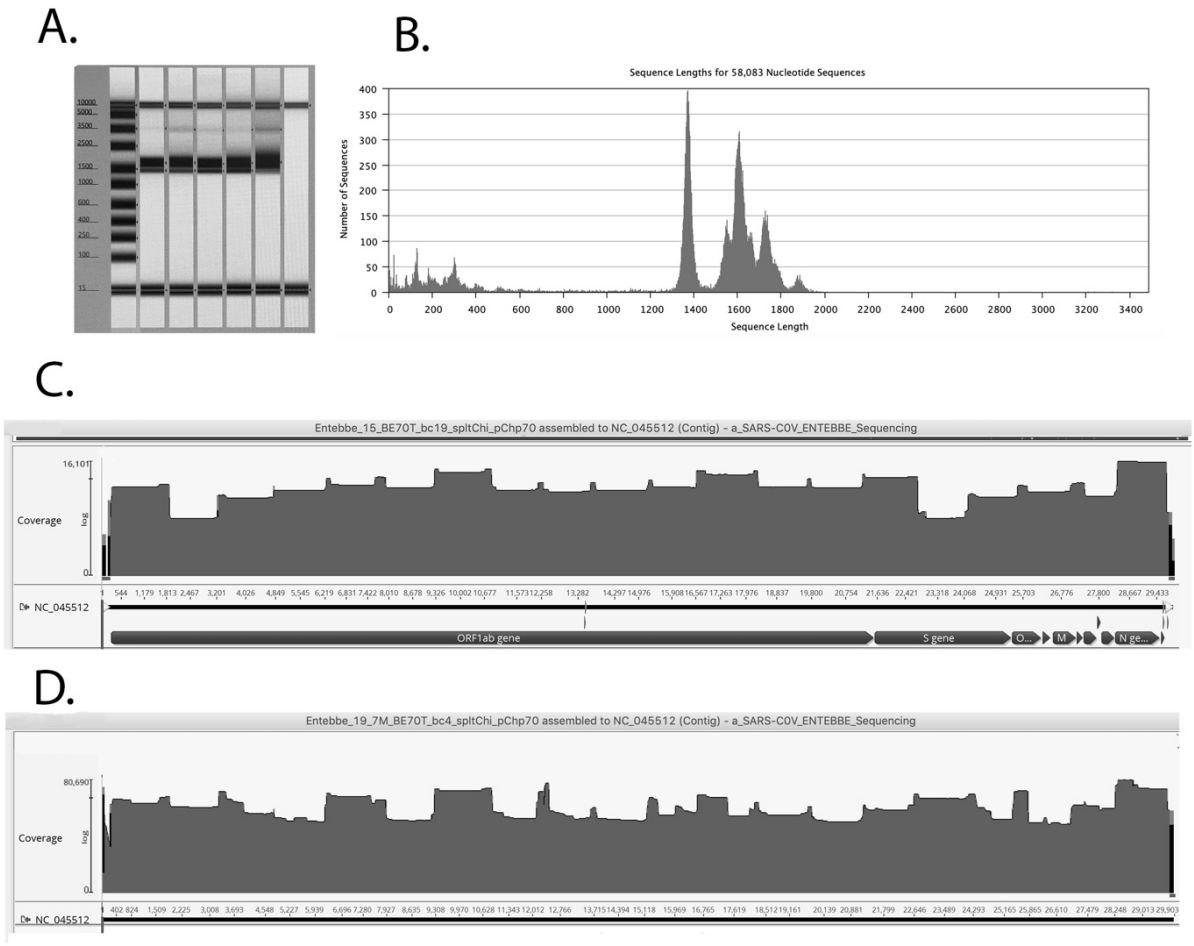

Figure 4. Testing the primer performance. Panel A, PCR product size after pooling of reaction A and B. Expected sizes of amplicons are from 1500 bp to 2093 bp before primer trimming. Panel B, MinION reads after quality control, primer, adapter trimming. Panel C, Reads mapped to SARS-CoV-2 reference genome, before amplicon 2 and 16 primer boosting. Panel D, Reads mapped to SARS-CoV-2 reference genome, after amplicon 2 and 16 primer boosting.

A set of SARS-CoV-2 clinical samples were tested with the Entebbe primers/protocol. Respiratory swab samples from 111 PCR confirmed cases of SARS-CoV-2 infection were processed for reverse transcription/PCR using the Entebbe primers and protocol as described in Supplementary Materials. If sufficient amplicon DNA was generated after PCR, MinION libraries were prepared, samples were sequenced on the MinION flowcells and the resulting data were assembled into genomes. Figure 5A shows the results

of this validation test. Complete genomes (fraction genome= 1) were obtained from samples up to Ct 35, 19 samples failed to yield sufficient amplicon DNA after PCR stage (figure 5A, red markers) and 5 samples yielded genomes with gaps > 842 nt. The PCR failures and the gapped genomes were not strictly associated with higher Cts and their distribution pattern is similar to the overall Ct distribution pattern across the set of samples (Figure 5 panel B) suggesting other factors such as sample quality, extraction method, storage, might be more critical than Ct in determining sequencing success, at least in samples within the Ct range tested (up to Ct 37).

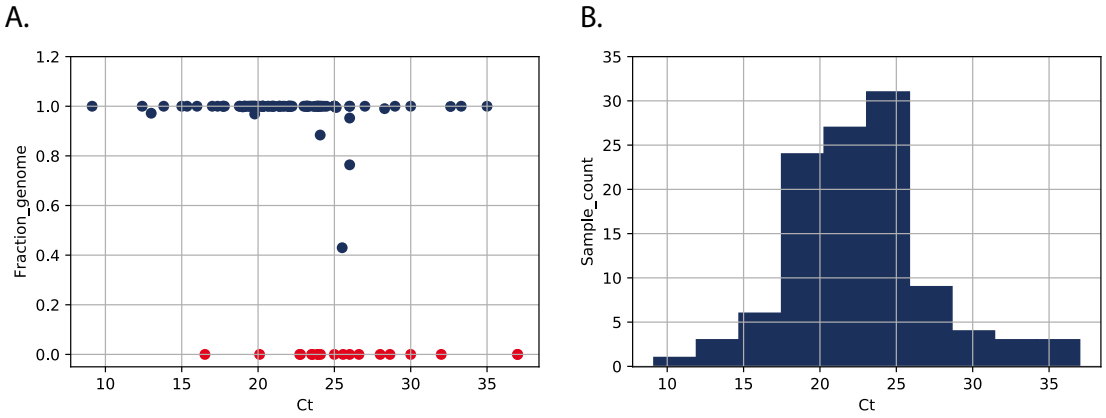

Figure 5. Validation of Entebbe primers. **Panel A** plots the genome yield (fraction of complete genome) as a function of sample Ct. Fraction genome was calculated by number of nonN nucleotides/29303 (the length, in nt, of NC_045512 reference genome). Each marker represents a sample, red markers indicate 19 samples that failed to yield sufficient DNA for library, 93 that proceeded to library preparation and sequencing (dark blue markers). **Panel B** is a histogram of the distribution of the 118 sample Cts.

Conclusions

Given the urgency of controlling the SARS-CoV-2 pandemic and the importance of having good quality SARS-CoV-2 genomes, we are providing these alternative primers (the Entebbe primers) with detailed step-by-step laboratory protocols to the community with the

hope that they benefit from the new design. The costs and efforts of sequencing SARS-CoV-2 in the large case numbers that are currently being seen are substantial and if these new primers result in a higher proportion of gap-less genomes, this will provide added value and will increase the utility of the resulting data.

Acknowledgements.

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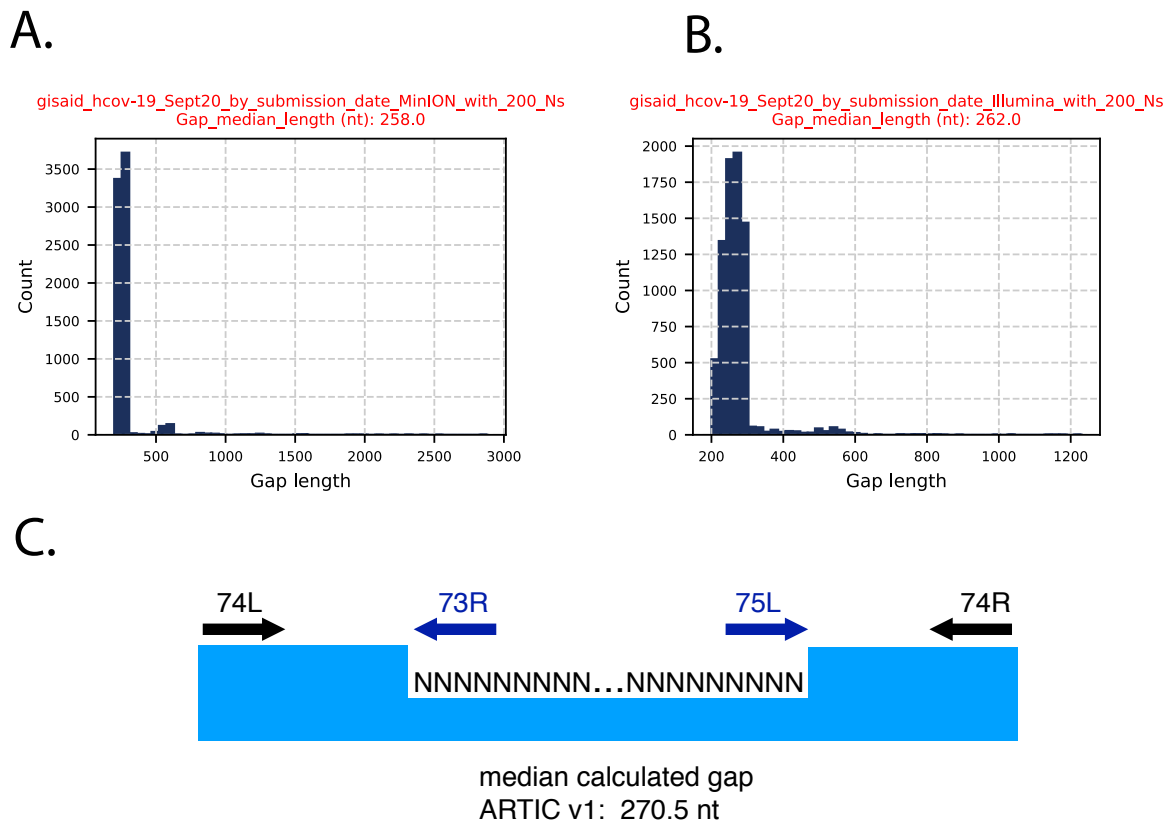
Supplementary Materials: 1. Supplementary Material Figure 1, 2. Detailed protocol, 3. Primer table.



Supplementary Material Figure 1. As described in Figure 1, SARS-CoV-2 genomes deposited in September 2020 (n= 38,228) were retrieved from GISAID, sorted by sequencing platform and the presence of 200N motifs was monitored. Panel A presents a histogram of gap lengths present in the first 3000 MinION-generated genome sequences that contained at least one 200N motif. The median length for all gaps was 258 nt. Panel B present the same analysis for Illumina-generated genome sequences. Panel C presents a typical gap. Because of overlapping amplicons, if a single amplicon is missing (amplicon 74 for example) from the sequencing library, the resulting gap in coverage would not be the complete amplicon 74 but would span from the 3' end of the adjacent amplicon 73 (after primer and quality trimming) to the 5' end of adjacent amplicon 75 (after primer and quality trimming). The calculated gaps generated by such amplicon loss have a median length of 270.5 nt, which is close to the the observed median gap length in the MinION data (258 nt) or Illumina data (262 nt) from September 2020.

2. Detailed protocol

We acknowledge the Oxford Nanopore Technologies and the ARTIC Network for their extensive protocols which provided important background details for the method presented here (19)(20). Potential users are strongly advised to study the original protocols carefully. The protocol presented here assumes the users has all the ARTIC and ONT background experience.

Detailed protocol for reverse transcription, PCR and sequencing library preparation.

Practical comments. Primers were ordered and delivered dry and were dissolved at 100 µM (= 100 pmol/µl) in PCR quality water. Dry shipped primers are reported to have better stability at room temperature (21). Ideal the primers were allowed to dissolve in PCR grade water for at least 3 hours at room temperature or overnight at 4 °C before diluting for use (see below).

Prepare the following primer mixes:

RPM_A: 21 primers. 3 µl of 100 µM stock of the following Reverse primers plus PCR grade water to 200 µl (= 300 pmol x 21 = 6300 pmol/200 µl = 32 pmol/µl. 2 µl per 20 µl RT reaction = 3.2 pmol/µl = 3.2 µM concentration in reaction) .

Primers in RPM_A: nCoV-2019_1_LEFT, AR1_3, AR1_4, AR3_11, AR3_12, AR5_19, AR5_20, AR7_27, AR7_28, AR9_35, AR9_36, AR11_43, AR11_44, AR13_51, AR13_52, AR15_59, AR15_60, AR17_67, AR17_68, AR19_75, AR19_76.

RPM_B: 21 primers. 3 µl of 100 µM stock of the following Reverse primers plus PCR grade water to 200 µl. (= 300 pmol x 21 = 6300 pmol/200 µl = 32 pmol/µl. 2 µl per 20 µl RT reaction = 3.2 pmol/µl = 3.2 µM concentration in reaction). **Primer Boost:** For Primers BR2_7, BR2_8, BR16_63, BR16_64 use 9 µl of 100 µM stock.

Primers in RPM_B: BR2_7, BR2_8, BR4_15, BR4_16, BR6_23, BR6_24, BR8_31, BR8_32, BR10_39, BR10_40, BR12_47, BR12_48, BR14_55, BR14_56, BR16_63, BR16_64, BR18_71, BR18_72, BR20_79, BR20_80, nCoV-2019_98_RIGHT.

PCR primer mix A (PPM_A): 1.5 µl of each primer (forward and reverse primer) plus 439 µl PCR grade water. = 41 primers (100 pmol/µl) = 4100 pmol in 500 µl water = 8.2 pmol/µl in the PPM. When 2 µl PPM used per 25 µl PCR reaction = 0.66 pmol/µl in reaction (= 660 nM).

Primers in PPM_A: nCoV-2019_1_LEFT, AF1_1, AF1_2, AR1_3, AR1_4, AF3_9, AF3_10, AR3_11, AR3_12, AF5_17, AF5_18, AR5_19, AR5_20, AF7_25, AF7_26, AR7_27, AR7_28, AF9_33, AF9_34, AR9_35, AR9_36, AF11_41, AF11_42, AR11_43, AR11_44, AF13_49, AF13_50, AR13_51, AR13_52, AF15_57, AF15_58, AR15_59, AR15_60, AF17_65, AF17_66, AR17_67, AR17_68, AF19_73, AF19_74, AR19_75, AR19_76 .

PCR primer mix B (PPM_B): 1.5 µl of each primer (forward and reverse primer) plus 439 µl PCR grade water = 41 primers (100 pmol/µl) = 4100 pmol in 500 µl water = 8.2 pmol/µl in the PPM. When 2 µl PPM used per 25 µl PCR reaction = 0.66 pmol/µl in reaction (= 660 nM). **Primer Boost:** for primers BF2_5, BF2_6, BR2_7, BR2_8, BF16_62, BF16_61, BR16_63, BR16_64, use 4.5 µl of 100 µM stock

Primers in PPM_B: BF2_5, BF2_6, BR2_7, BR2_8, BF4_13, BF4_14, BR4_15, BR4_16, BF6_21, BF6_22, BR6_23, BR6_24, BF8_29, BF8_30, BR8_31, BR8_32, BF10_37, BF10_38, BR10_39, BR10_40, BF12_45, BF12_46, BR12_47, BR12_48, BF14_53, BF14_54, BR14_55, BR14_56, BF16_61, BF16_62, BR16_63, BR16_64, BF18_69, BF18_70, BR18_71, BR18_72, BF20_77, BF20_78, BR20_79, BR20_80, nCoV-2019_98_RIGHT.

1. Set up Reverse Transcription reactions (two reactions per sample).

Mix the following components in a 0.2mL 8-strip tube, 1 RT_A and 1 RT-B reaction per sample.

Component	Volume (per sample)
RT_A_Reaction	
Component	**Volume**
RPM_A	1 µl
Template RNA	11µl (if using less than 11 µl viral RNA adjust to 11µl with water)
Total Volume	12µl
RT_B_Reaction	
Component	**Volume**
RPM_B	1 µl

Template RNA	11µl (if using less than 11 µl viral RNA adjust to 11µl with water)
Total Volume	12µl

Heat at 65 °C for 5:00 mins, and quickly chill in ice/water bath for at least 1:00 min.

Viral RNA input from a clinical sample should be between Ct 18-35. If Ct is between 12-15, then dilute the sample 100- fold in water, if between 15-18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition.

Reverse Transcription

Add the following to the 12 µl annealed template RNA sample:

Component	**Volume (1 reaction)**
SuperScript IV 5X Buffer	4 µl
100mM DTT	1 µl
RNaseOUT RNase Inhibitor	1 µl
10mM dNTPs mix (10mM each)	1µl
SuperScript IV	1 µl
(add 8 µl mix per reaction)	
Total Volume	20 µl

2. Incubate the RT reaction as follows:

42 °C 50 minutes

70 °C 10 minutes

Hold at 4 °C

3. Set up Amplicon PCR

In the Mastermix hood set up the multiplex PCR reactions as follows in 0.2mL 8-strip PCR tubes. Prepare one PPM_A and one PPM_B mix per sample.

Component	**1 reaction**	**8rxns_ PPM_A_Mix**	**8rxns_ PPM_B_Mix**
5 x Phusion HF buffer	5µl	40 µl	40 µl
10 mM dNTPs	0.5µl	4 µl	4 µl
Phusion DNA Pol	0.5µl	4 µl	4 µl
Primer PPM_A or PPM_B (10µM)	2 µl	16 µl	16 µl

Nuclease-free water	12 µl	96 µl	96 µl
Total	20 µl	per rxn	

Aliquot 20 µl per tube PPM_A_Mix or PPM_B_Mix

In the extraction and sample addition cabinet add **5µl cDNA** to each tube and mix well by pipetting.

4. Run PCR

Step	**Cycles**	**Temp**	**Time**
Heat Activation	1	98 °C	30sec
Denaturation	35	98 °C	15sec
Annealing	35	63 °C	30sec
Extension	35	72 °C	3 min
Polish	1	72 °C	10 min
Hold		4 °C	infinite

In post-PCR cabinet: After PCR, for each sample, pool the two reactions in a 1.5 ml microcentrifuge tube.

5. Clean up using AMPure XP beads, Agencourt.

Add an equal volume (1:1) of AMPure XP beads to the sample tube and mix gently by either flicking or pipetting. E.g. add 50 µl AMPure XP bead suspension to a 50 µl reaction.

Pulse centrifuge to collect all liquid at the bottom of the tube.

Incubate for 5 min at room temperature.

Place on magnetic rack and for 2 min

Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

Ethanol wash 1: Add 200µl of room-temperature 70%volume ethanol to the pellet.
Carefully remove and discard ethanol, being careful not to touch the bead pellet.
Repeat Ethanol wash
Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.

Open lid, allow to dry briefly (2 min)

Resuspend pellet in 30µl Elution Buffer (EB, QIAGEN)
Mix gently by flicking,
Incubate 5 min

Place on magnet and transfer sample (supernatant) to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.

6. Quantitate (A Tapestation 4200 analyzer with the D5000 assay kit as well as the Qubit fluorometer were used.)

7. Add Barcodes (Steps 7 onward derived from the Oxford Nanopore Technologies protocol (19)).

Set up the following reaction for each sample:

Component	**Volume**
DNA amplicons	5 µl*
Nuclease-free water	7.5 µl
Ultra II End Prep Reaction Buffer	1.75 µl
Ultra II End Prep Enzyme Mix	0.75 µl
Total Volume	15 µl

*Volumes usually varies based on the DNA concentration. For the 1500bp amplicons and pool size of appx 8 we have input 50ng and for a pool size of 12 we have input 20ng

Incubate at room temp (20°C) for 20 min
Incubate at 65 °C for 5:00 min
Incubate on ice for 1 min

E7645S/L NEBNext Quick Ligation Module

Component	**Volume**
End repaired sample	15µl
Barcode vol	2.5 µl
Ultra II Ligation Master Mix	17.5 µl
Ligation Enhancer	0.5 µl
Water	1.5 µl
final volume	35.5 µl

Incubate in a thermocycler for 15 minutes at 20°C with heated lid set to 30°C (or off).
Incubate at 70 °C for 10 min
Incubate on ice for 1 min

The 70°C incubation is to inactivate the DNA ligase to prevent barcode cross-ligation when reactions are pooled in the next step.

Pool all barcoded fragments together into a new 1.5 ml Eppendorf tube.

8. Clean up on AMPure XP beads, Agencourt.

Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. E.g. add 50 µl SPRI beads to a 50 µl reaction.

Pulse centrifuge to collect all liquid at the bottom of the tube.

Incubate for 5 min at room temperature.

Place on magnetic rack and for 5 min

Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

Ethanol wash 1: Add 200µl of room-temperature 70%volume ethanol to the pellet.
Carefully remove and discard ethanol, being careful not to touch the bead pellet.
Repeat Ethanol wash

Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.
Open lid, allow to dry briefly (2 min)

Resuspend pellet in 30µl Elution Buffer (EB, QIAGEN)
Mix gently by flicking

Incubate 5 min

Place on magnet and transfer sample (supernatant) to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.

9. Quantitate as above.

10. Add AMII adapters.

AMII adapters ligation reaction:

Component	**Volume**
Barcoded amplicon pools	30 µl
NEBNext (E60562)	
Quick Ligation Reaction Buffer (5X)	10 µl
AMII adapter mix	5 µl
Quick T4 DNA Ligase	5 µl
Total	50 µl

The input of barcoded amplicon pools will depend on the number of barcoded pools and should be between 40 ng (8 barcodes) and 160 ng (24 barcodes).

Incubate at room temperature for 15:00 min

11. Clean up on AMPure XP beads, Agencourt.

USE SFB instead of Ethanol for two washes ***CRUCIAL, Ethanol strips off motor protein from adapter. You don't want to do this!

Clean up on AMPure XP beads, Agencourt

Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. E.g. add 50 µl SPRI beads to a 50 µl reaction.

Pulse centrifuge to collect all liquid at the bottom of the tube.

Incubate for 5 min at room temperature.

598 Place on magnetic rack and for 2 min
599
600 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.
601
602 **SFB washes:** Add 200µl of room-temperature **SFB** to the pellet.
603 resuspend
604 collect on magnet
605 Carefully remove and discard **SFB** being careful not to touch the bead pellet.
606
607 **Repeat SFB wash**
608
609 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much
610 residual **SFB** as possible using a P10 pipette.
611
612 **Resuspend pellet in 15 *µ*l Elution Buffer (EB, QIAGEN)**
613 Mix gently by flicking,
614 **Incubate 5 min**
615 Place on magnet and transfer sample (supernatant) to a clean 1.5mL Eppendorf tube
616 ensuring no beads are transferred into this tube.
617
618 **12. Quantitate.**
619
620 **13. Proceed to MinION sequencing.**
621

622 **3. Supplementary material Table 1. Entebbe Primers**

Amplicon_no	Primer_id	Sequence[1]	Position[2]
1	AF1_1	GTCCGGGTGTGACCGAAAG	242
1	AF1_2	CCTTGTCCCTGGTTTCAACGA	274
1	AR1_3	TGCGGGAGAAAATTGATCGTACA	1894
1	AR1_4	GCACAGAATTTTGAGCAGTTTCA	1921
2	BF2_5	ACGTGCTAGCGCTAACATAGG	1540
2	BF2_6	GTTGGAGAAGGTTCCGAAGGT	1580
2	BR2_7	TAGCCTTATTTAAGGCTCCTGCAA	3477
2	BR2_8	TGCAACACCTCCTCCATGTTTAA	3459
3	AF3_9	ACAAACTGTTGGTCAACAAGACG	3220
3	AF3_10	CAACAAGACGGCAGTGAGGA	3233
3	AR3_11	TTGTGTAGATTGTCCAGAATAGGACC	4806
3	AR3_12	TCCATATGTCATTGACATGTCCACA	5014
4	BF4_13	TTTGGAAGAAGCTGCTCGGTATAT	4639
4	BF4_14	GAAACCATCTCACTTGCTGGTTC	4772
4	BR4_15	GGTTTTAGATCTTCGCAGGCAAG	6380
4	BR4_16	CCATTAGATCTGTGTGGCCAA	6534
5	AF5_17	CACACCCTCTTTTAAGAAAGGAGCTA	6190
5	AF5_18	TTGTTTGGCATGTTAACAATGCAAC	6234
5	AR5_19	CAGACGCTGATTTTGCAGATGA	7919
5	AR5_20	GCACTATCACCAACATCAGACAC	7994
6	BF6_21	AGGCTTTTGCAAACTACACAATTG	7591
6	BF6_22	TATGCTAATGGAGGTAAAGGCTTTTG	7574
6	BR6_23	CGATAGCTACAATACCACCAGCT	9409
6	BR6_24	CAATACCACCAGCTACTATAGATGCT	9397
7	AF7_25	AAGCTGGTGTTTGTGTATCTACTAGT	9243
7	AF7_26	ACCTACCTTGAAGGTTCTGTTAGAG	9164
7	AR7_27	CACTACCCAATATGGTACGTCCA	10885
7	AR7_28	TGAGTAACAACCAGTGGTGTGT	11000
8	BF8_29	CTGGAGTTCATGCTGGCACA	10560
8	BF8_30	GTTTTAGCTTGGTTGTACGCTG	10664
8	BR8_31	TTTGCCCTCTTGTCCTCAGATCTA	12313
8	BR8_32	TGGTATGACAACCATTAGTTTGGCT	12466
9	AF9_33	CTCAAGAAGCTTATGAGCAGGCT	12144
9	AF9_34	CAAGCTATAGCCTCAGAGTTTAGTTC	12089
9	AR9_35	ACGTTGACGTGATATATGTGGTACC	13770
9	AR9_36	ACGAGGTCTGCCATTGTGTATT	13802
10	BF10_37	GCGGTGTAAGTGCAGCC	13475
10	BF10_38	TCGCTTCCAAGAAAAGGACGAA	13602
10	BR10_39	CTCAATACTTGAGCACACTCATTAGC	15406
10	BR10_40	GTGACAAGCTACAACACGTTGT	15367
11	AF11_41	ATTCTATGGTGGTTGGCACAACA	15219
11	AF11_42	CAATAGCCGCCACTAGAGGAG	15173
11	AR11_43	TAGTGTAGGTGCACTTAATGGCATT	16932
11	AR11_44	GGTAAACAACAGCATCACCATAGTC	16846
12	BF12_45	AACATGTGACTGGACAAATGCTG	16566
12	BF12_46	ACTGACTTTAATGCAATTGCAACATG	16546
12	BR12_47	TACAGCAACTAGGTTAACACCTGTAG	18374
12	BR12_48	CACCCCTCGACATCGAAGC	18302

13	AF13_49	GTGGCAACTTTACAAGCTGAAAATG	18025
13	AF13_50	GACATACCTGGCATACCTAAGGAC	18160
13	AR13_51	GTTTAATGTTGCGCTTAGCCCAA	19791
13	AR13_52	GTAGTCCCAGATCACAGTATTAGCAG	19859
14	BF14_53	CGTGTATAACACGTTGCAATTTAGGT	19454
14	BF14_54	ACTTTGATGGACAACAGGGTGAA	19661
14	BR14_55	CGCGTGGTTTGCCAAGATAATTA	21285
14	BR14_56	CATAACCATCTATTTGTTCGCGTGG	21301
15	AF15_57	AGCTCATGGGACACTTCGC	21203
15	AF15_58	TTGGAGGTTCCGTGGCTATAAAG	21146
15	AR15_59	CGATTTGTCTGACTTCATCACCTC	22770
15	AR15_60	CTACCGGCCTGATAGATTTCAG	22971
16	BF16_61	TGCATCTGTTTATGCTTGGAACAG	22603
16	BF16_62	CTCTCTCAGAAACAAAGTGTACGTTG	22446
16	BR16_63	CACTTGCTGTGGAAGAAAGTGA	24371
16	BR16_64	GTTGACCACATCTTGAAGTTTTCCAA	24396
17	AF17_65	GGTGATTGCCTTGGTGATATTGC	24074
17	AF17_66	CTGTTTTGCCACCTTTGCTCA	24138
17	AR17_67	CCAGCAAAGAAAATAGTTGGCATC	25816
17	AR17_68	CGTAACAATTAGTATGCCAGCAAAGA	25830
18	BF18_69	TCCCTTTCGGATGGCTTATTGTT	25514
18	BF18_70	TGAAATCAAGGATGCTACTCCTTCAG	25446
18	BR18_71	GTTGTACCTCTAACACACTCTTGGTA	27451
18	BR18_72	CTCACAAGTAGCGAGTGTTATCAG	27418
19	AF19_73	ACGCTTTCTTATTACAAATTGGGAGC	27045
19	AF19_74	CGCTGTGACATCAAGGACCT	26994
19	AR19_75	TGGCAATGTTGTTCCTTGAGGAA	28755
19	AR19_76	CAGCCATTCTAGCAGGAGAAGTT	28885
20	BF20_77	TAGAGTATCATGACGTTCGTGTTGTT	28219
20	BF20_78	ACCCCGCATTACGTTTGGT	28309
20	BR20_79	TGGCTCTTTCAAGTCCTCCCT	29700
20	BR20_80	GCTCTTCCATATAGGCAGCTCTC	29779
	nCoV-2019_1_LEFT[3]	ACCAACCAACTTTCGATCTCTTGT	31
	nCoV-2019_98_RIGHT[3]	TTCTCCTAAGAAGCTATTAAAATCACATGG	29866

Footnotes 1. Sequence listed 5' to 3'.

2. Position in SARS-CoV-2 reference genome GenBank NC_045512

3. From original ARTIC primer set V.1 (20).